USGS

WATER RESOURCES RESEARCH GRANT PROPOSAL

Project Title: Ultrafiltration based detection of viruses and *Cryptosporidium* oocysts from environmental water samples.

Priority Problem Area: Water Quality

Focus Category: Water Quality, Methods

Keywords: Ultrafiltration, microbial concentration, Cryptosporidium oocysts, water quality, waterborne pathogens.

Duration: 12 months starting March 2000-February 2001.

2000 WRRI Award: Direct $25,000 Indirect $10,500 Total $35,500

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Congressional District Number. New Mexico II

Statement of Critical Water Resource Problem

In terms of drinking water safety, very little is known about the extent of viral and parasitic contaminants in source (influent) and product (finished) drinking water and their relationship to disease. Critical to both identifying and quantitating water-borne pathogens is the development and use of methods which reliably concentrate pathogens from drinking, surface and groundwater.

The Environmental Protection Agency (EPA) has mandated that large water utilities in the U.S. test their source and product water for viral pathogens for either surface or groundwater systems (EPA 1996). Although current methods for concentrating pathogens from these sources have allowed for the filtration of large volumes of water, there are difficulties in terms of 1) procedural complexity (time and expense), 2) variable efficiency and consistency of virus recovery and 3) use of different methods for detecting viruses, bacteria and protozoan organisms from water which increases the complexity and expense.

There is growing concern for the potential health risks associated with the presence of pathogens in surface, ground and drinking water however, little data is available to determine how significant these risks are. Improvement in the ability to document the prevalence and concentration of these pathogens in water from the standpoint of detection sensitivity, reproducibility and consistency of results will make it more feasible to obtain results that are more analyzable and cost effective.

Statement of Results, Benefits Expected

By the end of the funding period the following should be completed: **1)** Complete optimization for the recovery of viruses (T1, PP7 and poliovirus) and *Cryptosporidium* oocysts from spiked environmental water samples using field scale ultrafiltration systems. Determine the correlation between small scale and field scale testing of virus and oocyst recovery for the initial ultrafiltration step for concentrating these model organisms from environmental water, **2)** Optimization of processes downstream of the initial ultrafiltration step to allow for the concentration of the water sample to the final concentrate (5-40 ml). This would include processes for viruses as well as the integration of separation and concentration methods for *Cryptosporidium* oocysts, **3)** Complete optimization studies for integrating PCR assays for enterovirus and *Cryptosporidium* oocysts into the concentration and detection system (includes field testing), **4)** Field testing for the detection of bacteriophage (F specific RNA bacteriophage), enteroviruses and *Cryptosporidium* from tap, surface and ground water, **5)** If time allows begin initial tests to determine the sensitivity and reproducibility of other waterborne viruses such as Norwalk and adenovirus in small scale testing.

These results will provide the most detailed analysis of the use of ultrafiltration for concentrating microorganisms and the robustness of these systems to process field scale volumes in a relatively short period of time and allow for the processing of volumes that may be difficult to achieve by current methods. The results will also indicate whether the method being develop has advantages in terms of the detection of waterborne organisms by RT-PCR and PCR. These methods will not only be evaluated for recovery but the results will also be evaluated in terms of the expense, ease of use, speed of results and reproducibility of these methods compared to the standard method. In addition it is hoped that the small scale units will serve as good predictors of recoveries from field size systems. This will make testing of these systems with other organisms and in different water quality much more feasible. These results then have the potential to make a improvement on how microorgansims are concentrated and detected from large volumes of environmental water.

With this data, long-term support from sources such as the EPA, American Water Works Association Research Foundation, World Health Organization and filter manufacturers would be more feasible. There would be much more work that would need to be done before these methods were to be adopted as a standard method (other viruses, parasites, bacterial organisms, water conditions etc).

These agencies have supported research to determine the level of risk to the human population from waterborne viruses. Additional resources are needed to determine or in developing methods to provide needed information. In addition, there may be regional interest from the Southwest Center for Environmental Research and Policy (SCERP) and by the Waste-Management Education and Research Consortium (WERC). The long-term objective is to use this system to develop a series of studies to determine the relationship between the level of viral and protozoan contamination and documented cases of disease from these organisms as well as the integration of these systems for routine monitoring.

Nature, Scope and Objectives

The contamination of surface, ground and drinking water from viral, bacterial and parasitic organisms is a growing concern in developed and developing countries. The risk of waterborne viral agents to public health remains largely unknown due in large part to the difficulty in concentrating and detecting viruses from these environments both from a technical and practical standpoint and the associated high monetary costs involved in generating this information. Current methods to concentrate viruses have tended to be technically cumbersome (require a large number of steps) and have documented variable results based on differences in water quality and target viruses (see related research).

In response in part to the lack of information on the levels of viral pathogens in source and drinking water, The Information Collection Rule was initiated in 1997 by the EPA to mandate large utilities to collect and analyze water samples from their intake and finished product for viruses (EPA, 1996). In the future, there will continue to be a greater need for technologies that reliably concentrate and detect waterborne pathogens as water supplies are threatened by human activity.

This project is designed to improve the sensitivity and consistency of waterborne virus recovery and *Cryptosporidium* oocysts from surface, ground and drinking water as well as simplify the process of concentrating samples compared to the existing methods. Part of the objective is also to incorporate within the overall process, steps to optimize PCR detection of viral nucleic acids from the concentrate in terms of sensitivity, reproducibility, simplicity and cost. Efficient and cost effective methods for virus concentration and oocyst recovery can then be used to monitor the levels of viral contamination or to document the level of viral inactivation by the water treatment processes implemented by the utilities.

The overall objective is to use the results from small scale (2L) optimization experiments from the initial year of funding and apply these results as the bases for scale-up to field size (10-1000L) ultrafiltration systems. Optimized procedures for different types of water will be developed along with a single procedure that will efficiently recover pathogens consistently from all water types. Because these filters concentrate organisms based on size, the recovery of the larger, non-viral organisms are also feasible. This proposal will also determine the recovery efficiency for Cryptosporidium in field scale filtration systems to demonstrate the utility of using a single filtration step to concentrate viruses and larger organisms.

Objectives of this project.

1) Complete feasibility studies of field size ultrafiltration systems (hollow fiber and tangential flow, 10-1000L) as a first step concentration step in terms of:

A) Characterizing the recovery efficiency of infectious virus among different viruses (phage T1, PP7 and poliovirus) as well as Cryptosporidium oocysts from field scale samples.

B) Recovery efficiency with different water qualities (ground, surface, tap water).

C) Determine if the small scale filtration system is a good predictor of the performance of the field scale system.

2) Complete optimization of concentration procedures downstream of the initial ultrafiltration step. Process the concentrate to a final volume of 5-40 ml suitable for cell culture (IFA for *Cryptosporidium* oocysts) or PCR detection of virus or oocysts in the final concentrate.

3) Optimize RT-PCR detection of final concentrate for enterovirus detection and compare sensitivity to cell culture.

4) Optimize PCR detection of the final concentrate for oocyst recovery and compare detection to IFA.

5) Detection of naturally occurring enteroviruses and oocysts for field samples to verfy the optimized concentration and detection methods.

Methods, Procedures and Facilities

Background

By June of 2000 it is anticipated that field scale testing for the hollow fiber ultrafiltration system will be nearly complete and tangential flow systems will be at least halfway completed for filtering 100-1000 L depending on the type of water being filtered. We have had some delays in getting the field scale system on-line (delivery from the manufacturer was slower than anticipated, and some difficulty with parts etc.).

In the next funding cycle any remaining tests will be completed for tap, well and surface water. Initially 100 L of surface water, ground and tap water will be tested. Larger volumes of ground and tap water up to 1000L will also be tested for both ultrafiltration systems.

A prototype of the field scale ultrafiltration system has been developed and is currently being used for this project. This system has a centrifugal pump and can easily be adjusted to accommodate either a field scale hollow fiber or a tangential flow ultrafiltration system. The pump and filters are mounted on a cart so it is feasible to take into the field. Pump speed and transmembrane pressure regulation is used to control the input flow as well as the permeate flow rate. The objective is to have a sanitizable system that can process 10 -1000L in less than two hours.

For surface water samples, a initial prefiltration step has been developed to reduce fouling of the ultrafilter and remove large pieces of debris. Stainless steel sieves of 74, 58 and 36 um in series have been tested with the model viruses. Little virus was lost during this step when tested with Rio Grande water (< 5%) and prefiltration is very rapid. Early indications are that this will be sufficient to allow filtration of 100L of surface water from the Rio Grande for both the hollow fiber and tangential flow systems. Antibiotics and antifungal agents will be added to cell culture systems for poliovirus to further inhibit the growth of other microorganisms. Each experiment

will be done in triplicate. Treatment of the phage sample with ether in the final concentrate may be used if microbial contamination interferes with the plaque assay.

The hollow fiber system is currently being tested with 100L of ground or surface water with the two bacteriophages. Early results are very promising and have produced similar results as when done with 2 L samples (see progress review).

1) Complete feasibility studies of field size ultrafiltration systems (hollow fiber and tangential flow, 10-1000L) as a first step concentration step in terms of:

Water Quality

In the adsorption/elution method of virus concentration, the presence of organics in the water can compete for viral binding sites on the filter surface and lower virus recovery. Thus virus recoveries can be quite variable when microfiltration methods are used to concentrate viruses. We have tested several different water sources (tap, surface, ground) for its impact on recovery and flow rate on ultrafiltration in small scale experiments (see results section). The results do indicate under the appropriate conditions consistent recovery can be achieved among widely varying water qualities.

During the proposed funding period field scale tests for the recovery of phages T1 and PP7 in surface, ground and tap water will be completed. Limited challenges with poliovirus will also be done, however, because of the size of the challenges these will be kept to a minimum. Conditions that provide optimum recovery of phages T1 and PP7 will be used with poliovirus. Results from the small scale tests indicate that high recoveries with the phages do provide a good indication for efficient recovery of poliovirus. In all challenges after completion of each experiment all water and containers will be sanitized with overnight treatment with 100 ug/L sodium hypochloride before the water is returned to the sewage treatment system. The objective is to develop a single method that can efficiently recover viruses from tap, ground and surface water.

Virus Type

Viruses have been shown to have variable adsorption efficiencies based on differences in their surface chemistries (Guttmann-Bass and Armon 1983; Sobsey and Glass 1984). In this study, poliovirus, bacteriophages T1 and PP7 have been tested for recovery (Table 1). These viruses were selected for their ability to be assayed by cell culture, size differences and different surface characteristics and in the case of the phages, rapid results (one day assay). In addition, we have published data on the recovery of phages T1 and PP7 and poliovirus using the hollow fiber systems (Oshima et al. 1995a) and adsorption properties of these viruses with a number of different filter membranes (Oshima et al. 1994, 1995a,b and 1996). An important criteria for determining the effectiveness of ultrafiltration is to obtain similar high recoveries from each of these viruses regardless of water quality. The recoveries of poliovirus will be characterized by plaque assay and PCR to determine recovery efficiencies (determine the effect of PCR inhibitors). Bacteriophages T1 and PP7 utilize different bacterial hosts and can be co-challenged thereby providing more accurate comparison of recovery efficiency between the different bacteriophage. All phage T1 and PP7 experiments will be done as a duel challenge.

Table 1. Physical characteristics and host of model viruses to be used in this study.

Virus	Size	Host	Envelope	Nucleic Acid
Phage T1	50 nm head 150 nm tail	E.coli	No	dsDNA
Phage PP7	25 nm	P. aeruginosa	No	ssRNA
Poliovirus (Sabin 2 strain)	25 nm	HeLa	No	ss RNA

Blocking/Elution agents

For the large scale tests we have switched to calf serum instead of FBS (cost savings) as the blocking agent. The method that had the greatest promise in the small scale tests was the pretreatment of the filter module with 5% FBS followed with 0.05M glycine, pH 7.0 as a eluent at the end of the concentration step. This has been also tested in the field scale testing except calf serum will be used instead of FBS. Other methods may be tested if recoveries are insufficient. The final evaluation and selection of the optimized filtration condition will be based on a number of characteristics including: recovery efficiency, consistency between different viruses and water conditions, cost of the system, flow rate, ease of use and speed.

In this section of the study it will be determined which blocking/elution scheme provides the most sensitive and consistent recovery with the three model viruses. Consistent recovery between the different types of viruses will be established among the different sources of water (Table 2). This will be determined for both filtration systems. It will also be noted which methods produce the best results for each type of water. From these results it will be possible to determine how well the small scale ultrafiltration systems predict recovery from field scale systems.

Table 2. **Sample** table indicating the efficiency of recovery (percent recovery) of T1, PP7, and poliovirus from a 100 L sample. Results will be presented with various blocking agents if needed (nutrient broth, bovine serum albumin, fetal bovine serum, beef extract) with the hollow fiber and tangential flow ultrafitration systems. Three replicates per data point.

		Percent recovery with calf serum as the blocking agent		
	Filter system	Tap	Ground	Surface
T1	Hollow fiber			
	Tangential flow			
PP7	Hollow fiber			
	Tangential flow			
poliovirus	Hollow fiber			
	Tangential flow			

2) Optimization of processes downstream of the initial ultrafiltration step to allow for the concentration of the water sample to the final concentrate (5-40 ml). This would include processes for viruses and integration of separation and concentration methods for *Cryptosporidium* oocysts.

Objective: To develop a process to efficiently concentrate viruses to a final volume that can be assayed by either cell culture or RT-PCR

Sample processing will continue beyond the initial ultrafiltration process. Downstream processes will include a slow speed centrifugation step to remove particulates for viral samples (retenate volume is ~ 2L after the ultrafiltration step). The supernatant will be used for a second small scale ultrafiltration step (hollow fiber or tangential flow). The volume after this step will depend on the filtration system but should be between 20-100ml. Depending on the volume remaining concentration could terminate here or additional concentration methods such as spin column will be used to process the sample further to a volume < 5ml. Viral concentration will be determined after each step to monitor viral recovery throughout the process.

Other concentration steps such as organic flocculation or polyethylene glycol hydroextraction-dialysis may also be examined (American Public Health Association 1995). A second ultrafiltration step has the advantage of being faster (1-2 hr) and the recovery should be

predictable. Much data has already been generated on the filtration characteristics and virus recoveries using these small scale systems.

Field scale ultrafiltration (100-1000L)

Hollow fiber

Tangential flow

↓ < 2hrs

1-2L Centrifugation

(removal of much of the particulates)

30 min

↓

1-2L

Small scale ultrafiltration (hollow fiber) or

PEG precipitation or

flocculation

↓ <2 hr for ultrafiltration

25-100 ml

Spin column

30min

< 5ml

Detection of virus by cell culture and PCR

PCR detection

Figure 1. Overview of the ultrafiltration process and approximate time requirements to concentrate viruses from 100-1000L of water to <1 ml.

3) Recovery of poliovirus based on PCR amplification and detection of amplified product by ELISA.

Objective: to determine the efficiency of the detection of poliovirus by RT-PCR done on samples from the final retentate.

Large scale (100 L) samples from surface water (lower Rio Grande river) and ground water will be tested to optimize detection of polioviruses by PCR. A portion of the retentate from the samples that were used to determine the concentration of infectious poliovirus (see above) will be used to determine poliovirus concentration in the initial suspension and in the retentate by RT-PCR and detection of the amplified product by ELISA. Three replicate experiments will be done for the two water types (Table 3).

A Pan enterovirus primer set from the 5'noncoding region will be used to determine the efficiency of the PCR amplification (Muir et al. 1993). These primers amplify a 148 bp region. Detection of the amplified product will be by either agarose gel electrophoresis with ethidium bromide staining or by ELISA based detection (RNA/DNA hybrids) using a system that is currently under development in our laboratory (Figure 3). Tests with similar ELISA based detection with hepatitis B and C virus in Dr. Oshima's laboratory resulted in the routine detection of 10 targets per PCR reaction in plasma and cell culture fluid (Oshima et al. 1998). The detection sensitivity of the PCR assay will be compared with the same samples tested by plaque assay to determine the level of inhibition of the PCR reaction.

Table 3. **Example** of RT-PCR data to be generated from the recovery of spiked poliovirus nucleic acid after concentration from large scale volumes of water through the hollow fiber and tangential flow ultrafiltration systems and downstream processing steps. Three replicate

experiments per variable tested. Example poliovirus recovery when suspended in surface water with the filter blocked with calf serum and eluted with 0.05M glycine at pH. 7.0.

Exp.	Virus recovery unconcentrated sample	Virus recovery from the final concentrate	Minimum virus detection after spiking into the final concentrate[a]
1.	Concentration in the initial suspension	% recovery	minimum number of infectious viruses detected
2.			
3.			

[a] In separate experiments the sensitivity of the RT-PCR will be determined by adding known concentrations of poliovirus to the final concentrate (used as a indicator of the extent of PCR inhibition in the final concentrate and remaining in the sample handling process during RNA extraction). In these experiment virus is not added to the environmental sample until the final concentrate.

Similar results will be determined if other methods are used and for other types of water.

Figure 2. ELISA based detection of PCR product using a antibody molecule that detects RNA/DNA hybrids. (not available)

Recovery of *Cryptosporidium* oocysts

Very little work has been done to concentrate *Cryptosporidium* oocysts by ultrafiltration. A recent study by Juliano and Sobsey, 1997 has indicated the potential feasibility of hollow fiber ultrafiltration to concentrate oocysts from 50L of tap water (44%) and 10L of raw water (64%) with high concentrations of oocysts (10^5). In our laboratory, studies to recover *Cryptosporidium* using ultrafiltration was initiated by first developing a convenient and accurate method to get direct counts of low concentrations of oocysts from the initial suspension. This has been achieved by taking the initial suspension (with a PBS solution produce from reagent grade water) and filtering through a 13 mm polycarbonate 0.8 um filter and similarly taking the final retentate and filtering it through the 0.8um filter. In this way IFA detection of oocysts in both the input and retentate is feasible by probing, washing and detecting the oocysts directly on the 13 mm

membrane. This offers faster scanning of the complete disk and accurate counts of low numbers of fluorescing oocysts. This method then allows for accurate determination of low concentrations of oocysts in the initial suspension and in the final concentrate.

Field scale tests (10-1000L) will involve spiking studies of environmental water and isolation of naturally occurring oocysts from environmental water samples. The objective is to develop a process that can co-concentrate viruses and oocysts by the same method and allow for greater volumes of more turbid water to be sampled than in current methods. Initially methods that appeared to work well for the pencil module will be used. Three replicates will be done for each experimental condition. Low concentrations of oocysts will be used if possible however some high oocyst concentration will be used in some of the surface water samples. Processing of surface water samples will also integrate immunomagnetic separation (IMS) methods for separating sediments from oocysts or possibly sucrose or percoll gradients as well.
Oocysts will be detected and quantified using the indirect immunoflorecense assay (IFA) and by PCR. A PCR assay detecting a *Cryptosporidium parvum* specific primer set and downstream detection of the amplified product has been developed (Laxer 1991) and will be used to quantitate the oocysts concentrated through ultrafiltration. This system will be similar to the PAN enterovirus PCR detection method also being developed in our laboratory. The same ELISA detection method will be used for both assays. This will have the advantage of large scale PCR based detection methods that utilize the same ELISA based detection of the amplified product. ELISA based detection of PCR product from other waterborne pathogens can also be easily adapted to this system which is commercially available and requires no specialized equipment except for a ELISA reader.

Time Line

- Completion of the characterization and optimization of initial ultrafiltration step for phages T1 and PP7 and poliovirus from tap, well and surface water for the hollow fiber ultrafiltration system and/or the tangential flow system for field scale systems. Completed within the first 6 months July - Sept. 2000

- Integration of further concentration steps downstream of the initial ultrafiltration step. Optimization for recovery of infectious virus and by PCR. Oct. - April, 2001

- Completion of the characterization and optimization of initial ultrafiltration step for *Cryptosporidium* oocysts from tap, well and surface water for the hollow fiber ultrafiltration and/or the tangential flow system for field scale volumes. July - October, 2000

- The recovery efficiency of oocysts concentrated by ultrafiltration and detected by PCR. November - April, 2001

- Field testing of ultrafiltration system (Rio Grande) for enteroviruses and Cryptosporidium May-June, 2000.

Facilities

My laboratory has the cell culture facilities (biosafety cabinets, CO_2 incubators) needed for virus culture and viral plaque assays. In addition, Pall Corporation (a major filter manufacturer) will be providing hollow fiber and tangential flow hardware needed to handle 100-1000L including filters and pumps for this project ($30,000). The laboratory also has equipment for PCR amplification and detection of the amplified product. Thus there is no anticipated need for any large items of equipment.

Related Research

The most commonly used method for the recovery of viruses from water involves the adsorption of viral particles on a 0.2 - 0.45 μm filter (typically a electropositive membrane) and the subsequent elution using beef extract (American Public Health Association 1995; Goyal and Gerba 1982). Elution of adsorbed viral particles is the result of the competition for adsorptive sites between the proteins in beef extract and the viral particles. Since there is an over abundance of protein compared to virus the protein out competes for adsorptive sites. Virus recovery with the adsorption/elution method can be variable depending on water quality, virus type and the laboratory which performed the assay (Melnick et al. 1984). Once the virus is eluted, virus can be further concentrated by a number of concentration methods including polyethylene glycol (PEG), organic flocculation, dialysis and spin column chromatography.

A number of variables affect the ability of virus to adsorb to filter membranes. These include organic compounds in the water adsorbing to the filter membrane, the binding of organic material with viruses to inhibit adsorption or the organic load inhibiting efficient elution. Variables that affect adsorption include organic acids, proteins, cations and filter surfaces themselves (Guttman-Bass and Catalano-Sherman 1985; Sobsey and Glass 1984; Oshima et al. 1995; Rose et al. 1984; Schwab et al. 1995; Shields and Farrah 1983; Sobsey and Glass 1984; Sobsey and Hickey 1985).

Less information is available on virus recovery by ultrafiltration. Several studies with ultrafilters have been done to recover human viruses (Belfort et al. 1975a and b; Berman et al. 1980). However these studies did not examine effects of using different viruses and water qualities and did not examine the effect of ultrafiltration on the efficiency of PCR detection systems for waterborne viruses. More recently, ultrafiltration has also been used for the second step concentration procedure (after adsorption/elution) for recovery from small volume of hepatitis A virus and poliovirus (Divizia et al. 1989). Concentration of marine bacteriophage was achieved via ultrafiltration after prefiltration through 0.2 and 0.1 μm filters for <0.5 L volumes Wommack et al. 1995). To date, a systematic approach (different filters, viruses, water qualities, blocking agents, effect on PCR) to determining the effectiveness of ultrafiltration as a first step and possibly second step virus concentration procedure has not been done.

Special sample processing concerns arise when PCR is used as the detection method. Inhibitors to the PCR can also be concentrated with the viral particles (Tsai and Olson 1992). Elution can result in a increase in inhibitors such as humic acid. Removal of these inhibitors has been found

to enhance the sensitivity of the PCR (Schwab et al. 1995; Shieh et al. 1995). Ultrafiltration may have advantages in reducing the concentration of inhibitors in the concentrated sample.

In recent years there has been increased concern over the presence of Cryptosporidium in surface, ground and drinking water (LeChevallier et al. 1991; Rose et al. 1991; Hancock et al. 1998). Cryptosporidium oocysts are notoriously difficult to concentrate. In a evaluation of commercial laboratories in the USA 6 of 12 laboratories failed to detect seeded oocysts and the average percent recovery was only 2.8% (Clancy et al. 1994). Other studies have also report high variability and poor recovery (Nieminski et al. 1995; LeChevallier et al. 1995).

This suggests there is room for improvement of the basic methodology so that consistent recovery is achieved even though water quality and target organisms may vary. Simplification of the process will also aid in improving the consistency of results and lowering the time and cost of processing samples. The goal is to have a method which allow for consistent recovery between different locations (water qualities) and different target organisms even when conducted by different laboratories. Ultrafiltration offers a alternative to existing method that can provide more consistent recovery between organisms and because size exclusion is the mechanism for concentration all microorganisms may be concentrated by single method.

Progress Review

Virus Stability

The following is a summary of what has been accomplished thus far. The initial phase of this project was to first establish the stability of the model viruses in suspension fluids that will be used in the ultrafiltration experiments and then to conduct experiments that would characterize the recovery of virus from a regent water where the recovery of virus is most straight forward and easy to characterize. Maintaining the stability of the model viruses for the duration of the ultrafiltration steps is needed in order to accurately assess the efficiency of virus concentration. Experiments with ultrapure water and in phosphate buffered saline (PBS) indicated that maintenance of viral infectivity was better with PBS than with ultrapure water. These experiments were done with virus (~1000 PFU/ml) suspended in ultrapure water and PBS. Stability was measured in terms of PFU/ml at 0, 1, 3 and 24 hrs incubation at room temperature (results not shown). From these results all recovery experiments were made with virus suspended in PBS.

Recovery of Virus from Reagent Water Using Ultrafiltration.

Pretreatment with Blocking Agents to prevent adsorption of viruses to the filter (Hollow Fiber System).

Without any treatments to either prevent viral adsorption and/or the elution of adsorbed virus from the filter, low recovery of all three model viruses was observed. Pretreatment of the ultrafilter by blocking of the membrane surface with proteinaous materials appears to prevent viral particles from binding to the filter during concentration. Several a were tested (nutrient

broth, beef extract, BSA or FBS) to block adsorption of viruses to the filter before the concentration of the 2L sample was begun.

In all recovery experiments a 2 L virus suspension was concentrated down to 30-50 ml (hold up volume of the system). The concentration of virus in the initial virus suspension (2 L) was compared to the total infectious virus recovered in the retentate. After some preliminary testing on methods to block the membrane and maintain high flow rates after blocking the method of choice was to make the solution of blocking material in 200 ml. This blocking solution was then recirculated through the hollow fibers in the cross flow mode without any backpressure such that no fluid passed through to the permeate (see Figure 1). The fluid was circulated in this manner for at least 1 hr at room temperature. After 1 hr the blocking agent was removed and the system was flushed with ultrapure water to remove any unbound blocking material prior to the introduction of the 2 liter virus suspension.

Results from the hollow fiber ultrafiltration system indicated that all blocking agents had a positive effect on the efficiency of virus recovery compared to filters that were not pretreated (Table 4). The most efficient recovery was using a 1% FBS solution as a blocking agent. Recovery was similar when a lower concentration of virus was used (~10 PFU/ml) with phages T1 and PP7 and the hollow fiber blocked with 2% nutrient broth (results not shown).

Table 4. Efficiency of virus recovery from 2 L of phosphate buffered saline with different pretreatment agents using a hollow fiber, polyacrylonitrile 50,000 MWCO ultrafilter. All experiments are expressed as the mean value of three experiments. Standard deviation indicated inside parenthesis.

Blocking Agent		Mean % virus recovery (+/- standard deviation)		Permeate Flux[a] (ml/min)	Time[b] (min)
No Blocking Agent[c]	T1	1.7	(1.1)	161	12
	PP7	4.8	(5.4)	161	12
	Polio	ND[d]			
No Blocking Agent	T1	22	(25.3)	180	10
	PP7	38	(30)	180	10
	Polio	4.4	(4.8)	127	15
2% Nutrient Broth	T1	58	(7.2)	140	13

	PP7	91	(11.8)	140	13
	Polio	52	(24.6)	137	15
4% Nutrient Broth	T1	69	(39.2)	135	15
	PP7	100	(48.1)	135	15
	Polio	28	(17.7)	108	19
5% BSA	T1	40	(22.0)	51	45
	PP7	97.6	(6.8)	51	45
	Polio	57	(41.6)	30	70
1% Beef Extract	T1	12	(20.0)	188	11
	PP7	29	(46.2)	188	11
	Polio	ND [d]			
5% Beef Extract	T1	47	(31.6)	144	15
	PP7	79	(41.0)	144	15
	Polio	43	(18.5)	153	13
1% Fetal Bovine	T1	47	(10.1)	87	25
Serum	PP7	94	(20.3)	87	25
	polio	98	(6.6)	72	28

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[a] Permeate flux at the end of filtration.

[b] Time to complete filtration.

[c] Reagent grade water

[d] Not done.

Recovery efficiency of the tangential flow ultrafiltration system after pretreatment with blocking agents.

Based on the results from the hollow fiber system, a smaller regiment of blocking agents were tested with the tangential flow system (MWCO of 10,000 Da) (Table 5). The results indicate that recovery also improved with the addition of a blocking step for the tangential flow system. For both systems, the highest recovery appeared to be when 1% FBS was used as a blocking agent.

Table 5. Efficiency of virus recovery with different blocking agents using a 10,000 MWCO polyethercarbonate tangential flow ultrafilter. Virus was first suspended in 2 liters of phosphate buffered saline and concentrated ~100 ml. All experiments are expressed as the mean value of three experiments. Standard deviation indicated inside parenthesis.

Blocking Agent		Mean % virus recovery (+/- standard deviation)		Permeate Flux[a] (ml/min)	Time[b] (min)
2% Nutrient Broth	T1	57	(8.7)	45	25
	PP7	65	(14.2)	45	25
	Polio	53	(6.9)	64	35
4% Nutrient Broth	T1	68	(20.9)	52	55
	PP7	68	(19.1)	52	55
	Polio	19	(7.2)	38	63
5% Beef Extract	T1	40	(12.8)	68	31
	PP7	91	(8.7)	68	31
	Polio	15	(2.3)	60	36
1% Fetal Bovine Serum	T1	52	(18.0)	102	18
	PP7	87	(12.0)	102	18
	Polio	74	(10.6)	60	35

[a] Permeate flux at the end of filtration.

[b] Time to complete filtration.

[c] Not done.

Recovery of Virus from Environmental Water (Tap, Well, Surface) Using Ultrafiltration.

Based on the results from earlier experiments, 1% FBS appeared to produce the best recovery and was used to recovery viruses from ground and surface water (Tables 4 and 5). Therefore 1% FBS was used as the initial method to optimize virus recovery from environmental water. In addition to 1% FBS, 5% FBS and increasing the duration for the blocking step to overnight was also tested because 1% FBS did not perform as well in well water (Table 6).

Pretreatment of the hollow fiber ultrafilter with 5% FBS appeared to be beneficial virus suspended in well water but did not provide adequate recovery from surface water perhaps because of viral particles in surface water binding to particulates and organics in surface water especially as the sample is concentrated in the retentate (Table 6).

Table 6. Recovery of phages T1 and PP7 using different blocking agents and water sources using the hollow fiber system. Average recovery for 3 replicate experiments.

Blocking Agent	Water Type		Mean % virus recovery (+/- standard deviation)		Permeate Flux[a] (ml/min)	Time[b] (min)
1% FBS	Tap	T1	46	(24)	95	32
		PP7	78	(17.5)	95	32
	Well	T1	12	(2.3)	63	37
		PP7	37	(7.8)	63	37
5% FBS	Well	T1	72	(17)	63	37
		PP7	68	(12.2)	63	37
	Rio Grande	T1	6	(3.3)	29	74
		PP7	32	(9.5)	29	74

5% FBS	Rio Grande	T1	17	(7.9)	37	63
	Prefiltered	PP7	13	(7.2)	37	63
	11um					

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[a] Permeate flux and the end of filtration.

[b] Time to complete filtration.

To further improve recovery from surface water, additional treatments were examined. In one set of experiments 0.5% of FBS was added to the initial 2L virus suspension. Recovery however was quite variable depending on the virus and the filtration rate was reduced. Other treatments included the addition of 0.5% FBS to the retentate after 1,500 ml was collected in the permeate. This was done to increase the filtration rate and simplify the procedure (no pretreatment step was done). In addition, a more traditional elution using 0.05M glycine at pH. 7.0 was added as an elution agent in ultrafilters that were pretreated with 5% FBS as a blocking agent (Table 7). These results indicate a marked improvement in recovery.

Recovery of viruses using the tangential flow filtration system from 2 L of environmental water produced similar results as with the hollow fiber with a few notable differences. The use of glycine as a elution step did not provide improved recovery and the recirculation of the retentate reduced recovery. In addition, the tangential flow system appears provide better permeate flow in highly turbid surface water compared to the hollow fiber system although the recovery may be somewhat lower (Tables 6 and 7). Both systems in small scale filtration systems appear to have utility for testing with field scale systems.

These results indicate for surface water with high turbidity both prevention of virus adsorption to the filter and to particulates in the water sample is important to achieve high recoveries of virus. Treatments such as 0.5% FBS added near the end of the concentration or the use of 0.05M glycine as an eluent improved recovery. The use of 0.5% FBS or similar complex proteinaous agent can be a simple and cost effective method to obtain efficient recovery of virus from all types of water while maintaining adequate permeate flux.

These conditions will be tested in a field size system using a much larger module (membrane surface area 59 times greater than the pencil module). A even larger module that is 276 times larger is also available. Assuming a direct scale up these field size modules (comparison of surface areas) and comparable concentration factors between the two systems a flow rate of 221 L/2hr for the pilot scale ($1m^2$ membrane area) or 1035 L/2hr for the largest module ($1m^2$ membrane area) for the Rio Grande water is anticipated. Initially the smaller module will be examined because of the smaller holdup volume (300 ml). The largest module has a holdup volume of 1,200 ml. The total hold-up volume will be higher than noted when the volume required for the additional plumbing is included for each system. The volume targeted is 100L for surface water, ~500 L for ground and 1000L for drinking water. These volumes reflect the

filterability of each type of water, the relative levels of microbial contaminants found in each type of water and what volumes are commonly collected in these types of water.

Table 7. Recovery of phages T1 and PP7 from different waters using the hollow fiber system. Average recovery for 3 replicate experiments.

Treatment	Water Type		Mean % virus recovery (+/- standard deviation) Immediate	w recirc	Time[b] (min)
0.5% FBS	Ground	T1	28 (7)	**57[c] (22)**	58
500ml		PP7	38 (6)	**61[c] (13)**	58
Polio 71 (16)			**90[c] (7)**	44	
Elution with	Ground	T1	2 (1)	14 (9)	37
0.05 M glycine		PP7	2 (0)	23 (11)	37
pH 7.0					
5% FBS	Ground	T1	24 (16)	**87 (3)**	102
Block with		PP7	38 (6)	**88 (23)**	102
elution 0.05M glycine		Polio	21 (25)	**90 (7)**	87
pH. 7.0					
0.5% FBS	Surface	T1	38 (12)	ND	127
addedto 2L		PP7	78 (21)	ND	127
		Polio	21 (11)	ND	121
0.5% FBS	Surface	T1	7 (4)	**27[d] (8)**	64
added last		PP7	12 (6)	**51[d] (4)**	64
500ml		Polio	15 (16)	**81[c] (3)**	55
5% FBS	Surface	T1	33 (33)	**61 (13)**	59
Block with		PP7	41 (23)	**85 (3)**	59
elution 0.05M glycine,		Polio	10 (9)	**82 (12)**	106
pH 7.0					

[a] Permeate flux and the end of filtration.
[b] Time to complete filtration.
[c] 30 min recirculation of retentate prior to virus assay
[d] 15 min recirculation of retentate prior to virus assay

Table 7. Characteristics of the hollow fiber and tangential flow ultrafiltration modules.

Module	Module	Membrane	Module (Feed Side)

	Length (mm)	Diameter	Area m^2	Hold-up Volume (ml)
Hollow Fiber				
Pencil	130	20	0.017	9
Pilot	552	60	1.0	300
Production	1,129	89	4.7	1,200
Tangential Flow				
Centramate			0.092-0.46	24-120
Centrasette			0.46-2.32	120-600

Table 8. Recovery of phages T1 and PP7 and poliovirus from different waters using a 10 000 MWCO polyethersulfone tangential flow ultrafiltration system. Each data point is the mean of three replicate experiments.

Treatment	Water Type		Mean % virus recovery (+/- standard deviation) Immediate	w recirc.[b]	Time[a] (min)
0.5% FBS added to last 500 ml	Ground	T1	65 (22)	21 (16)	13
		PP7	81 (16)	43 (10)	13
0.5% FBS	Surface	T1	24 (13)	18 (18)	15

added		PP7	51 (14)	41 (19)		15
to last 500 ml						
5% FBS	Ground	T1	**63 (27)**	61 (19)		17
block with		PP7	**77 (35)**	72 (20)		17
glycine elution 0.05M		polio	**43 (10)**	ND		18
5% FBS	Surface	T1	**53 (2)**	36 (15)		22
block with		PP7	**92 (17)**	55 (22)		22
glycine elution 0.05M		polio	**52 (13)**	ND		21
5% FBS	Surface[d]	T1	**51 (13)**	ND		
		PP7	**81 (25)**	ND		
		polio[e]	**77 (10)**	ND		26

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[a] Time to complete filtration.

[b] 30 min recirculation of retentate prior to virus assay. In some cases refers to the recirculation of the elution agent.

[c] Experiment done once.

[d] No prefiltration. Reflects the recovery of phage with the use of a elution agent (100 ml) containing 0.05 M glycine and 0.5% FBS (recirculated across the membrane for 5 min) then the eluent was added to the retentate.

[e] Virus bound to the membrane was eluted with the recirculation of 100 ml, 0.05% FBS in 0.05M glycine pH. 7.0 for 5 min. This was then combined with the retentate to determine the % recovery.

Field Scale Ultrafiltration

Recently, field scale (100L) virus recovery experiments have been conducted with the hollow fiber system (ground water, T1 65%, PP7 71% n=3) ; surface water (T1 80%; PP7 90% n=2). Recovery has been similar to results from the small scale tests (Table 9). Filtration of 100 L was completed in 47 min for ground water and 135 min for surface water (Rio Grande, 12 NTU). These results indicate the hollow fiber system is capable of filtering and recovering surface water

samples with minimal prefiltration. Additional adjustments should result in even faster filtration times and perhaps increased recoveries.

Based on results from the small scale tests of the tangential flow system it is expected that it will take less time to process 100L with this system and should accommodate more turbidity than using the hollow fiber ultrafilter (~100 min).

Table 9. Optimal recovery of phages T1 and PP7 and poliovirus with a hollow fiber 50,000 MWCO polyethersulfone ultrafilter from 2L water samples. Most efficient concentration methods are in bold.

Blocking Agent	Water Type	% virus recovery [a] Phage			Time min. [c]
		T1 (S.D.)[b]	PP7 (S.D.)[b]	Poliovirus (S.D.)[b]	
5% calf serum with 0.05M glycine elution	Ground 100L[f]	2.3 (1) **71[H] (10)**	5 (2) **70 (15)**	ND	47, ND
5% calf serum with 0.05M glycine elution	Surface 100L[f]	ND **76[H]**	ND **80**	ND ND	135, ND

[a] Average virus recovery for three replicate experiments. [b]Standard Deviation

[c] Time to concentrate 2L suspension of virus to the holdup volume. [d] Membrane blocked with 5% FBS an elution agent before concentration was initiated, bound virus was eluted with 0.05M glycine buffer was added to the retentate and recirculated for 30 min. [e]Raw water prefiltered with 11 um filter before use. [f]100 lL surface water 2 replicate experiments, prefiltered through 75, 48 and 36 um screens (12 NTU for surface water). [g]0.5% FBS added after initial 2 L volume was concentrated to 500 ml and the final retentate recirculated for 15 or 30 min. [h] recovery after the elution step.

Pan enterovirus PCR detection of concentrated virus

Development of a PAN enterovirus RT-PCR assay has been completed. With this system the PCR product is detected by ELISA. Optimization with poliovirus 2 and 3 indicates the sensitivity to be ~0.6 -0.06 PFU/PCR tube. Initial tests with concentrated surface water detected poliovirus 2 at the same detection sensitivity as stock virus. This sample was concentrated from 100L to 40 ml by using a hollow fiber ultrafilter followed by centrifugation and a second ultrafiltration with a small scale system. This concentrate was then spiked with known amounts of poliovirus 2 and the viral RNA extracted and amplified via RT-PCR. Initial tests indicate sensitivities that are comparable to stock virus detection (minimal inhibitors) of 0.6-0.06 PFU/PCR tube. These result

suggests that ultrafiltration, downstream concentration methods and the extraction-RT-PCR detection systems produces a concentrated virus sample where RT-PCR can be done very efficiently. Typically much more complex procedures are employed before the extraction step to remove PCR inhibitors. These results suggest that the concentration procedure may have advantages with improved PCR detection perhaps by introducing fewer inhibitors and/or the process removes inhibitors. The extraction procedure that is utilized has a spin column step that should be helpful in removing inhibitors prior to the RT-PCR step. Additional tests with field scale concentration of viruses from environmental water will further verify our preliminary results.

Recovery of *Cryptosporidium*

Ultrafiltration membranes retain viral particles by size exclusion and should therefore have pore sizes that would be able to also efficiently retain *Cryptosprodium* oocysts (1-5 um) and other microorganisms. Thus it is feasible that a single ultrafiltration system could be adapted to recover all organisms using a single method which could replace the current multiple method approach for these organisms. Low concentrations of *Cryptosporidium* oocysts was spiked into 2L volumes (4 oocysts/ml) of reagent, tap, ground or surface water to determine the recovery efficiency using the hollow fiber ultrafiltration system. Efficient recoveries have been observed that appear to be independent of water quality as hoped (Table 10). These recoveries indicate the potential feasibility of this system to recover viruses and Cryptosporidium simultaneously. These results were tested with conditions that are similar to recoveries for viruses indicating the feasibility for using the initial ultrafiltration step to concentrate viruses and oocysts.

Table 10. Recovery efficiencies (%) of *Cryptosporidium* oocysts from 2 L of deionized, tap, ground and surface water using a 50,000 MWCO hollow fiber ultrafilter. Membranes treated with 10% SDS between uses and blocked with 5% FBS prior to use.

# of Replicates	Water type	Turbidity (NTU)	Ave # of Oocysts Oocysts (SD)[a]	Mean % Recovery (SD)
4	Deionized [b]	0.00	7933 (784)	47.8 (3.1)
3	Deionized [b,c]	0.00	0 (0)	0.0
3	Tap	0.11	613 (45)	65.0 (9.9)
3	Well	ND	888 (466)	75.8 (9.4)
3	Arkansas R.	1.42	866 (225)	76.6 (6.2)
3	Rio Grande	30.9	201,000 (12,238) [d]	81.0 (11.4)

[a] Average number of oocysts spiked into the 2L sample.

[b]Membranes not blocked or treated with SDS.

[c]Carry over control (see if oocysts remained in the filtration system after sanitation).

[d]Oocyst concentration was increased in order to detect oocysts by IFA off of a 13 mm disk (retentate was diluted to reduce sediment concentration in the retentate).

Cryptosporidium oocyst detection by PCR.

A similar PCR assay has been developed for Cryptosporidium using a region that appears to be specific for *C. parvum* (Laxer et al. 1991). Optimization tests indicate sensitivity of 1 oocyst/PCR reaction from purified oocysts. Oocyst detection by PCR from concentrates will continue to be evaluated.

Work to be completed in remaining funding period (June 30, 2000)

1) Much of the field scale testing for the hollow fiber ultrafiltration and some of the tangential flow systems will be completed for viruses.

2) Frame work for downstream processing for virus concentration will be completed (determination of what processes are the most efficient). Complete characterization with replicates may not be completed.

3) Begun to optimization of PAN enterovirus RT-PCR on the final retenate.

4) Begin to examine recoveries of Cryptosporidium oocysts from 10-100L samples.

Training Opportunities as a Result of WRRI Funding

During the course of this study numerous opportunities for training of graduate and undergraduate students took place. Much of the results from the first year of funding was presented at the American Society for Microbiology annual meeting in Atlanta in May 1998 by Ann Ommani as part of her research towards completing a Master's degree (completed December 1998). Research was also presented at the ASM branch meeting in January 1999 and will be presented at ASM in May 2000. A manuscript has been submitted for publication thats describes the results of small scale testing of ultrafiltration systems to concentrate water-borne viruses. A manuscript of Cryptosporidium oocyst recoveries using the hollow fiber ultrafiltration system is almost completed.

Linda Winona a master's student has continued the small scale ultrafiltration studies since the departure of Ms. Ommani. Ms. Winona will conduct the scale up studies.

In addition this project has provided supplies for John Olszewski, a Ph.D. candidate who is developing a pan enterovirus PCR that utilizes detection of the amplified product by ELISA. This assay will be used in the downstream enterovirus detection after the initial ultrafiltration step. In collaboration with Digene Diagnostics, a enzyme immunosorbant assay (ELISA) will be developed which will allow for rapid and economical detection of the PCR product. This will be used to determine if there are benefits to the use of ultrafiltration in improving the efficiency of the detection of viruses by PCR by either improving the sensitivity of detection (minimizing the effect of inhibitors to PCR) and/or simplification of the steps needed to conduct PCR assays.

Summer research opportunities were provided for three undergraduate students as part of an ongoing research education for undergraduates (REU) program within the biology department.

These students conducted the initial experiments on the developing filtration conditions for the tangential ultrafiltration system.

Summary of accomplishments

1) Virus stability tests completed.

2) Initial characterization in clean water system for hollow fiber and tangential flow

system completed.

3) Tests in small scale ultrafiltration system in environmental water completed for the 3 viruses and Cryptosporidium oocysts for 2 L volumes. Several potential methods for scale-up identified.

4) Prototype field scale system designed and delivered.

5) Testing of hollow fiber and tangential flow systems for 100L samples for the 3 viruses have been initiated.

6) RT-PCR assay and ELISA detection has been optimized for poliovirus.

7) PCR assay for Cryptosporidium oocysts has been optimized.

8) Downstream processes for viruses from field scale (100L) has been initiated.

Presentations/Publications

Results presented as American Society for Microbiology May 1999.

Results presented as American Society for Microbiology May 1998.

Results presented at WRRI meeting September 1998.

Results presented at Rio Grande ASM Branch meeting January of 1999.

Results presented at ASM General meeting May 1999.

Manuscript submitted November 1999 entitled “Efficient and predictable recovery of viruses from water by small scale ultrafiltration systems”

Manuscript will be submitted shortly entitled “Small scale hollow fiber ultrafiltration of Cryptosporidium oocysts from water”

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